

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
23 May 2002 (23.05.2002)

PCT

(10) International Publication Number  
WO 02/40630 A2

(51) International Patent Classification<sup>7</sup>: C12N

CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(21) International Application Number: PCT/IL01/01060

(22) International Filing Date:  
15 November 2001 (15.11.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
139708 15 November 2000 (15.11.2000) IL

(71) Applicant and

(72) Inventor: AMIEL, Gilad, E. [IL/IL]; 32a Ehud Street, 34553 Haifa (IL).

(74) Agent: G. E. EHRLICH (1995) LTD.; 28 Bezalel Street, 52521 Ramat Gan (IL).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PROCESS OF DECELLULARIZING BIOLOGICAL MATRICES AND ACELLULAR BIOLOGICAL MATRICES USEFUL IN TISSUE ENGINEERING

(57) Abstract: A method of decellularizing a biological matrix so as to obtain an acellular biological matrix, as well as clinical uses of the acellular biological matrix are disclosed. The method is effected by (a) obtaining a tissue of interest; (b) treating the tissue of interest with a decellularization solution containing a cell lysis reagent, preferably a combination of a non-ionic detergent with a mild base; (c) removing cell remnants, thereby obtaining the acellular biological matrix; and optionally (d) dehydrating the acellular biological matrix.

BEST AVAILABLE COPY

PROCESS OF DECELLULARIZING BIOLOGICAL MATRICES AND  
ACELLULAR BIOLOGICAL MATRICES USEFUL IN TISSUE  
ENGINEERING

5 FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to processes of decellularizing biological matrices; novel acellular biological matrices obtained thereby; artificial organ/tissue implants prepared therefrom; and their use in corrective medicine. More particularly, the present invention relates to  
10 processes of decellularizing biological matrices derived from two-dimensional tissues/organs; novel two-dimensional acellular biological matrices obtained thereby; artificial tubular, bladder-like or planar implants prepared therefrom; and their medical use in replacing, augmenting and/or repairing tubular, bladder-like or planar  
15 malfunctioning, injured, diseased or absent tissues or organs, or portions thereof, in a subject in need thereof.

Wherever applicable, autologous native tissue of one functionality and of lesser essentially is used to replace lost or malfunctioning tissue having another, more essential, functionality. For example, saphenous  
20 veins are used for coronary artery bypass grafting. Gastrointestinal segments are used to replace the esophagus, ureters and/or the bladder. Cartilage is removed from joints and ribs, and reimplanted in other damaged joints or in the trachea. Skin grafts are currently an essential component in the treatment of extensive burns so as to reduce scarring in  
25 exposed body portions. Bone grafts are used to correct hearing defects. Many more examples exist.

However, *in-vivo*, *ex-situ*, native tissues rarely replace the entire function of the original lost tissue and bear the risk of future complications, including metabolic abnormalities, infections, perforation  
30 and malignancy. In addition, the limited amount and repertoire of

autologous native tissues available for such procedures limits the possibilities for such reconstructions.

Synthetic prostheses such as silicone, polyvinyl and TEFLON are also widely used in recent years for reconstructing or bypassing damaged, missing or diseased tissue. Synthetic prostheses bear the risk of causing a wide range of complications, including device malfunction, infections, thrombosis and stone formation, in addition to mechanical and biocompatibility problems.

Tissue engineering is a fairly new approach to tissue reconstruction. Initially, cells were harvested, expanded *in-vitro* and re-implanted in target organs. However, the injection of cell suspensions directly into target organs has a low success rate due to the anchorage-dependent characteristics of the majority of mammalian cells. Furthermore, three-dimensional structures for implantation require a pre-designed scaffold, which serves as a substrate for cell-adhesion.

New functional tissues are reconstructed by transplantation of cells onto biological or synthetic biocompatible materials. These scaffolds serve as an extracellular matrix for cell seeding and tissue reconstruction. The native extracellular matrix is known to assist in arranging cell of different types into functional tissue arrangement, controls tissue structure and, at times, controls cell phenotype. Synthetic materials and biomaterials are the primary classes of materials presently employed in tissue engineering.

***Synthetic materials:***

TEFLON and DACRON are two of the more popular synthetic materials presently available for the substitution of biological structures. These materials do not aim to resemble the basic structure of extracellular matrices, they are non-degradable, they do not enhance tissue regeneration, and possess a very limited potential for cell adherence thereto. Therefore, they are limited to the substitution of large diameter blood vessels or as

patches in the cardiovascular and other systems. Synthetic materials expose the patient to an increased risk of infection, foreign body reaction and inflammation, thrombosis or occlusion and implanted device failure.

***Biomaterials:***

5 Naturally derived materials such as collagen and alginate, synthetic biodegradable polymers such as polyglycolic acid (PGA) and polylactic acid (PLA) and acellular (decellularized) tissue matrices such as small-intestinal submucosa and bladder submucosa are the three major classes of biomaterials that have been widely used for tissue engineering.

10 ***Naturally derived materials:***

Collagen is the most abundant protein in the mammalian body and can be easily extracted from tissues with enzyme treatment and salt/acid extraction [Li (1995) *Biologic Biomaterials: tissue-derived bio-materials (collagen)*. In: Bronzo JD (ed) *The biomedical Engineering Handbook*. CRC, Boca Raton, Florida, pp 627-647]. Due to its minimal antigenic nature [Furthmayr *et al.*, *Int Rev Connect Tissue Res* 7:61-99, 1976; DeLustro *et al.*, *Clin Orthop* 260:263-79, 1990], collagen was approved for various medical applications in the U.S. by the Food and Drug Administration (FDA), including wound dressing and artificial skin implants [Pachence *et al.*, *J Biomed Mater Res* 33:35-40, 1996]. When implanted, collagen tends to degrade due to lysosomal attack. However, most presently available collagen products undergo cross-linking, which reduces their degradation rate. Although collagen can be processed into a wide variety of structures, such as sponges, fibers and films [Cavallaro *et al.*, *Biotechnol Bioeng* 43:781-791, 1994; Li (1995) *Biologic Biomaterials: tissue-derived bio-materials (collagen)*. In: Bronzo JD (ed) *The biomedical Engineering Handbook*. CRC, Boca Raton, Florida, pp 627-647], Yannas *et al.*, *J Biomed Mater Res* 14:65-81, 1980], it does not resemble the native three-dimensional structure of native extracellular matrices. In addition,

cross-linking of collagen is inhibitory to cell adhesion [Middelkoop *et al.*, Cell Tissue Res 280:447-53, 1995].

Alginate is a biocompatible polysaccharide isolated from seaweed [Smidsrod *et al.*, Trends Biotechnol 8: 71-78, 1990]. Since it is inert and possesses limited mechanical properties, its use is mainly limited to acting as a cell delivery vehicle [Smidsrod *et al.*, Trends Biotechnol 8: 71-78, 1990]. Alginate is approved by the FDA for use as a wound-dressing material.

***Synthetic biodegradable polymers:***

PGA, PLA and other polymers form a group of alpha-hydroxy acids containing polyesters that are widely used in medicine, especially as suture materials. Since the degradation rate, the three-dimensional structure, the porosity and the configuration into fiber mesh of these materials can be controlled [Freed *et al.*, Biotechnology 12: 689-693, 1994], they have gained popularity in tissue engineering research. However, these synthetic polymers lack biological recognition. Therefore, tissue remodeling and function is limited during the biodegradation of these materials, leading to disappointing long-term results [Niklason *et al.*, Science 284:489-93, 1999].

***Acellular matrices:***

Acellular (decellularized) matrices are naturally derived scaffolds useful in tissue engineering. Decellularizing attempts have been described for many structures. Presently, small intestine submucosa [Badylak, Presented at the 1<sup>st</sup> SIS Symposium, Orlando, Florida 11-12/12/1996; Badylak *et al.*, J Surg Res 47:74-80, 1989; Lantz *et al.*, J Invest Surg 3:217-227, 1990; Lantz *et al.*, J Surg Res 53: 175-181, 1992; Lantz *et al.*, J Invest Surg 6: 297-310, 1993; Prevel *et al.*, Ann Plast Surg 35: 374-380, 1995] and bladder submucosa [Chen *et al.*, Urology 54: 407-410, 1999; Dahms *et al.*, Urology 50: 818-825, 1997; Piechota *et al.*, J Urol 159: 1717-1724, 1998; Probst *et al.*, Br J Urol 79: 505-515, 1997] are the most thoroughly investigated. Various decellularizing procedures have been

Current modalities for tissue engineering of small caliber tubes as constructs for implantation are far from being satisfactory. Blood vessels and ureters provide the two more extensively studies examples, as follows:

*Ureters:*

5       The gastrointestinal tract is currently the mainstay source of tissue for bladder and urethral reconstruction or replacement. Malignancy, stone formation and congenital anomalies are the common causes requiring the interposition of intestinal segments. However, bowel is associated with many side effects secondary to the loss of the urothelium-urine barrier  
10 [Merguerian World J Urol 18: 31-35, 2000]. Acid-base electrolyte imbalances, disorders of calcium-phosphate metabolism, stone formation, dysuria-hematuria in cases of gastrocystoplasty, chronic bacteruria, perforation, malignant transformation and excessive mucous formation are some of the reported complications.

15       Various attempts to create an urothelium-lined substitute have been published in the scientific and medical literature. Collagen tubular sponges seeded with urothelial cells have been used to replace ureteral segments in dogs. No muscle regeneration into the graft was evident in these experiments. Severe stricture formation and papillary mucosal  
20 thickening was noted at the anastomotic site in addition to severe salt deposits along the graft after 12 weeks [Tachibana et al., J Urol 133: 866-869, 1985]. Ureteral acellular matrices were previously used to replace ureteral segments in rats [Dahms et al., Urology 50: 818-825, 1997]. Although epithelialization and smooth muscle infiltration was  
25 observed into the matrix, it was noted that acellular matrices treated with 1 % sodium azide followed by 2000 Kuntz units DNase that were implanted in various areas in the urinary tract developed urinary calculi in 85 % of the grafted animals [Sievert et al., World J Urol 18: 19-25, 2000]. Sutherland *et al.* reported a 63 % of stone formation in grafted animals  
30 when acellular matrices originating from allograft stomach and bladder

were used for bladder augmentation in a rat model [Sutherland et al., J Urol 156:571-576, 1996]. The variability of the type of stones, including struvite (60-100 %) indicates that chronic infection was also present in the grafts.

5        ***Blood vessels:***

Diseases of the small and medium caliber arteries, led to the interest in the surgical treatment of vascular disease, which account for the majority of deaths in the United States each year [Weinberg et al., Science 231:397-400, 1986]. Over 400,000 coronary bypass grafts and 50,000  
10    peripheral bypass grafts are performed annually [Heart & Stroke Facts: American Heart Association. <http://www.americanheart.org/statistics>. (1996)]. However, up to 30 % of the patients who require arterial bypass surgery do not possess suitable or sufficient saphenous veins of the leg, which remains the standard conduit for coronary bypass surgery [Edwards  
15    et al., Surg Gynecol & Obstet 122:37-42, 1966]. Furthermore, it has become evident that the long-term patency of vein grafts is poor [Campeau et al., Circulation 52:369-377, 1975; Chesebro et al., N Eng J Med 307:73-78, 1982; FitzGibbon et al., Circulation 57: 1070-1074, 1978; FitzGibbon et al., J Am Coll Cardiol 17: 1075-1080, 1991]. Since these  
20    operations have been conducted for the last 3 decades, it was uncovered that many of the patients require a second and third operation. In addition to the increased morbidity and mortality associated with reoperations, some patients are restricted to medicinal treatment due to shortage of suitable conduits for arterial replacement [Ivert et al., Scand J Thorac  
25    Cardiovasc Surg 22: 111-118, 1988; Nair et al., J Cardiovasc Surg 30: 656-660, 1989; Dougenis et al., Eur Heart J 13: 9-14, 1992]. In peripheral vascular repair, the most widely used synthetic grafts, i.e., polytetrafluoroethylene or DACRON (polyethylene terephthalate fiber) for large caliber high blood-flow areas are compromised by thrombogenicity  
30    or anastomotic intimal hyperplasia leading to stenosis in small caliber

and/or other low flow sites, such as below the knee [Stephen et al., Surgery 81: 314-318, 1977; O'Donnell et al., J Vasc Surg 1:136-148, 1984].

In the last two decades many attempts have been made to engineer patent small caliber (< 5-6 mm) arterial substitutes. Weinberg and Bell were the first to report an attempt to engineer a blood vessel substitute by seeding endothelial cells, smooth muscle cells and fibroblasts on pre-formed collagen gels [Weinberg et al., Science 231: 397-400, 1986]. Unfortunately, mechanical and burst strength were too poor to allow its implantation *in-vivo*. In recent years, novel approaches for producing small-caliber arterial grafts have been proposed. L'Heureux *et al.* introduced a method based exclusively on the use of cultured cells without an external synthetic or biological matrix support [L'Heureux *et al.*, FASEB J 12: 47-56, 1998]. Human vascular smooth muscle cells cultured with ascorbic acid produced a cohesive cellular sheet, which was placed around a tubular support to produce the media of a vessel. A similar sheet of human fibroblasts was wrapped around the media to provide the adventitia. Thereafter, endothelial cells were seeded in the lumen thus formed. *In-vivo* studies in dogs revealed a 50 % patency rate after 7 days. Niklason *et al.* described seeding smooth muscle cells and endothelial cells on biodegradable polymer made of polyglycolic acid [Niklason et al., Science 284: 489-493, 1999]. *In-vivo* studies have demonstrated patency of the grafts up to 26 days. Badylak *et al.* was the first to introduce the concept of small intestinal submucosa as a vascular graft [Badylak et al., J Surg Res 47: 74-80, 1989]. Huynh *et al.* introduced yet another model, utilizing an acellular collagen matrix as a small caliber graft [Huynh et al., Nat Biotech 17: 1083-1086, 1999]. Their analysis showed that the lumen of the grafts was endothelialized in within 3 months, which contributed to the long-term patency in a rabbit model.



However, transanastomotic endothelialization of synthetic grafts in humans has proven to be limited, questioning the clinical applicability of animal models, which tend to endothelialize grafts rapidly [Davids et al., in Tissue engineering of prosthetic vascular grafts. (eds Zilla & Griesler) 3-45 (RG Landes Co, Austin TX; 1999; Berger et al., Ann Surg 172: 118-127, 1972; Sauvage et al., Arch Surg 109: 698-705, 19]. It has been shown that a confluent endothelial cell monolayer on small-caliber prosthetic grafts may provide immediate protection from thrombus formation following patent implantation [Anderson et al., Surgery 101: 577-586, 1986; Sentissi et al., Surgery 99: 337-42, 1986; Jarrell et al., Ann Surg 203: 671-678, 1986]. However, this protection does not occur in humans. Current sources of human endothelial cells, such as human umbilical vein endothelial cells have been studied extensively [Schneider et al., Surgery 103:456-462, 1988]. However, these cells cannot serve as a source of endothelial cells due to immune incompatibility. Readily available human endothelial cells for vascular grafts from autologous sources have been poorly explored.

Due to the current limitations of decellularized two-dimensional grafts in general and small caliber vessels in particular, a need was identified to refine the current decellularization techniques in order to preserve as much as possible the original structure of the extracellular matrix, in addition to exploring new methods to elevate the smoothness of the surface of the decellularized constructs to avoid thrombogenicity, hyperplasia, plaque and stone formation, strictures and to reduce the risk of infection.

While reducing the present invention to practice, a novel decellularization process of porcine arteries, ureters and other tissue and organs was developed to yield a collagen rich acellular matrix. To evaluate the potential of autologous cell adherence to the new matrix, endothelial cells were harvested from short segments of Human Saphenous

Vein (HSVEC) and cultured *in-vitro*. Cells of other origins were also harvested and cultured. Subsequently, cells were seeded on the matrix to form a monolayer. This approach offers an off-the-shelf readily available matrix seeded with autologous cells as a feasible approach to the formation of a small caliber blood vessel, as well as other structures. The decellularization method integrates a unique and highly advantageous sequence of chemical and mechanical treatments to harvested tissue. It allows the conservation of the original arrangement and mechanical properties of the extracellular matrix characterizing the original tissue. In addition, it provides an excellent scaffold for harvested cells to be reimplanted *in-vivo*. Upon implantation, the acellular matrix is anticipated to support cell ingrowth and regeneration, to degrade slowly and to eventually be replaced by native extracellular matrix proteins.

#### 15 SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a method of decellularizing a biological matrix so as to obtain an acellular biological matrix, the method comprising the steps of (a) obtaining a tissue of interest; (b) treating the tissue of interest with a decellularization solution containing a cell lysis reagent; (c) removing cell remnants, thereby obtaining the acellular biological matrix; and optionally (d) dehydrating the acellular biological matrix.

According to further features in preferred embodiments of the invention described below, prior to step (b) the tissue of interest is treated under hypotonic conditions sufficient to lyse red blood cells, yet which maintain extracellular matrix architecture.

According to still further features in the described preferred embodiments the hypotonic conditions include immersion in distilled water at 2-42 °C, preferably about 4 °C for less than 2 hours.

According to still further features in the described preferred embodiments the cell lysis reagent includes at least one non-ionic detergent.

According to still further features in the described preferred  
5 embodiments the cell lysis reagent includes at least one mild base.

According to still further features in the described preferred  
embodiments the non-ionic detergent is selected from the group consisting  
of TRITON-X 100, TWEEN 20, TWEEN 40, TWEEN 80, tetraethylene  
glycol monoethyl ether, heptaethylene glycol monododecyl ether, sucrose  
10 monolaurate, polyoxyethylene 20 cetyl ether (Brij 58), polyoxyethylene 23  
lauryl ether (Brij 35), octanoyl-N-methylglucamide (MEGA-8), N-octyl  
beta-D-glucopyranoside (OGP), lauryl maltoside (DDM) and PEG 600.

According to still further features in the described preferred  
embodiments the non-ionic detergent is employed at a concentration of 0.5  
15 - 2.5 volume %.

According to still further features in the described preferred  
embodiments the mild base is selected from the group consisting of a mild  
hydroxide base and a mild non-hydroxide base.

According to still further features in the described preferred  
20 embodiments the mild hydroxide base is selected from the group  
consisting of ammonium hydroxide, trimethylammonium hydroxide,  
triethanolammonium hydroxide, monoethanolammonium hydroxide, and  
benzylammonium hydroxide.

According to still further features in the described preferred  
25 embodiments the mild non-hydroxide base is selected from the group  
consisting of sodium acetate, sodium benzoate, sodium propionate and  
sodium phenoxide.

According to still further features in the described preferred  
embodiments the mild hydroxide base is employed at a final concentration

of 10-100 mM, preferably about 50 mM, derived from a stock solution of say about 1 M.

According to still further features in the described preferred embodiments steps (b) and (c) are performed under substantially isotonic conditions.

According to still further features in the described preferred embodiments step (c) is effected by repeated washes with an isotonic solution.

According to still further features in the described preferred embodiments steps (b) and (c) are repeated in sequence at least twice.

According to still further features in the described preferred embodiments step (b) is accompanied by shaking.

According to still further features in the described preferred embodiments step (c) is accompanied by shaking.

According to still further features in the described preferred embodiments step (b) is performed at 4-42 °C.

According to still further features in the described preferred embodiments step (b) is continued or repeated until a glistening white appearance of collagen is apparent and a sponge-like texture is obtained.

According to still further features in the described preferred embodiments step (b) is performed for at least 24 hours.

According to still further features in the described preferred embodiments step (b) is performed for about 48 hours.

According to still further features in the described preferred embodiments the step of dehydrating the acellular biological matrix is effected by baking.

According to still further features in the described preferred embodiments the step of dehydrating the acellular biological matrix is effected by freeze-drying.

According to still further features in the described preferred embodiments the step of dehydrating the acellular biological matrix is effected by lyophilization.

5 According to still further features in the described preferred embodiments the step of dehydrating the acellular biological matrix is effected at underpressure.

According to still further features in the described preferred embodiments the tissue is a tubular tissue, whereas prior to the step of dehydrating the acellular biological matrix, the acellular biological matrix  
10 is mounted over a cylindrical element.

According to still further features in the described preferred embodiments the method further comprising the step of removing the acellular biological matrix from the cylindrical element.

According to still further features in the described preferred  
15 embodiments the cylindrical element has a smooth surface and it is preferably a silicone or TEFLON tube.

According to still further features in the described preferred embodiments the tissue of interest is derived from a gastrointestinal tract of an animal.

20 According to still further features in the described preferred embodiments the tissue is selected from the group consisting of neo-esophagus, neo-stomach, small bowel, large bowel, biliary tract, a pancreatic duct and a rectum.

According to still further features in the described preferred  
25 embodiments the tissue of interest is derived from a cardiovascular system of an animal.

According to still further features in the described preferred embodiments the tissue is selected from the group consisting of an artery, a vein, a heart valve and a heart wall.

According to still further features in the described preferred embodiments the tissue of interest is derived from a genitourinary system of an animal.

5 According to still further features in the described preferred embodiments the tissue is selected from the group consisting of a renal collecting system, a ureter, a kidney, a bladder, a urethra, a testis, a testis sac and a penis.

According to still further features in the described preferred embodiments the tissue of interest is derived from a skin of an animal.

10 According to still further features in the described preferred embodiments the tissue of interest is derived from an epidermis of an animal.

According to still further features in the described preferred embodiments the tissue of interest is derived from an endodermis of an animal.

15 According to still further features in the described preferred embodiments the tissue of interest is derived from a mesoderm of an animal.

According to still further features in the described preferred embodiments the tissue of interest is derived from a parenchymal structure of an animal.

20 According to still further features in the described preferred embodiments the parenchymal structure is selected from the group consisting of kidney and liver.

25 According to still further features in the described preferred embodiments the tissue of interest is derived from a muscle structure of an animal.

According to still further features in the described preferred embodiments the muscle structure is selected from the group consisting of a heart muscle, a smooth muscle, and a skeletal muscle.

30

According to still further features in the described preferred embodiments the tissue of interest is derived from a skeletal structure of an animal.

According to still further features in the described preferred  
5 embodiments the skeletal structure is selected from the group consisting of a cartilage, a tendon and a bone.

According to still further features in the described preferred embodiments the tissue of interest is derived from a nervous system of an animal.

10 According to still further features in the described preferred embodiments the tissue is selected from the group consisting of a dura and a nerve.

According to still further features in the described preferred embodiments the tissue of interest is derived from a two-dimensional  
15 organ of an animal.

According to still further features in the described preferred embodiments the tissue of interest is derived from a three-dimensional organ of an animal.

According to still further features in the described preferred  
20 embodiments the tissue of interest is derived from a human being.

According to still further features in the described preferred embodiments the tissue of interest is derived from a mammal.

According to still further features in the described preferred embodiments the mammal is a pig.

25 According to another aspect of the present invention there is provided an acellular matrix obtained by any one or combination of the embodiments of the method described herein above. The matrix may be rehydrated and supplemented with one or more growth factors, such as, but not limited to, VEGF and it's various isoforms, Basic FGF, EGF,

PDGF, TGF-beta, IGF, HBGF and Angiopoietins and their various isoforms.

According to yet another aspect of the present invention there is provided a method of preparing an artificial implant for replacing, augmenting, bypassing or repairing a malfunctioning, injured, diseased or absent tissue, organ or a portion thereof, in a subject, the method comprising the steps of (a) obtaining an acellular biological matrix using any one or combination of the embodiments of the method described herein above; and (b) seeding at least one cell type of interest on the acellular biological matrix.

According to further features in preferred embodiments of the invention described below, the at least one cell type of interest is autologous.

According to still further features in the described preferred embodiments the at least one cell type of interest is selected from the group consisting of endothelial cells originating from arteries, veins or capillaries; fibroblast cells; dermal cells, including, but not limited to, keratinocytes, squamous cells, basal cells, melanocytes; different types of epithelial cells, such as urothelial cells, oral bucal cells, tracheal broncheal cells; hormone-endocrine secreting cells; hematopoietic stem cells (either from bone marrow or circulating), embryonal stem cells; gastrointestinal mucosal cells, including without limitation, parietal cells, mucous cells, chief cells and enteroendocrine cells; smooth muscle cells, cardiac muscle cells, skeletal muscle cells; nervous system cells, including glial cells, satellite cells, astrocytes, oligodendrocytes; bone cells, including osteoblasts, osteocytes, osteoclasts, chondrocytes, hepatic cells including kupffer cells, endothelial cells, fat storing cells, sinusoidal capillary cells, renal cells, including renal derived epithelial, stromal and endothelial cells and hair follicle cells. According to still another aspect of the present



invention there is provided an artificial implant prepared using any one or combination of the embodiment the method described above.

According to an additional aspect of the present invention there is provided a method of replacing, augmenting, bypassing or repairing a malfunctioning, injured, diseased or absent tissue, organ or a portion thereof, in a subject, the method comprising the steps of (a) obtaining the acellular biological matrix using any one or combination of the embodiments of the method described herein above; and (b) implanting the acellular biological matrix in the subject.

According to still an additional aspect of the present invention there is provided a method of replacing, augmenting, bypassing or repairing a malfunctioning, injured, diseased or absent tissue, organ or a portion thereof, in a subject, the method comprising the steps of (a) obtaining an artificial implant using any one or combination of the embodiments of the method described herein above; and (b) implanting the artificial implant in the subject.

The present invention successfully addresses the shortcomings of the presently known configurations by providing a new method of decellularizing biological matrices to thereby obtain acellular biological matrices of superior properties which are used for the preparation of superior artificial organ/tissue implants which are efficiently usable in corrective medicine.

#### BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood

description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIGs. 1a-b are light microscope photographs, magnified 1:250 and 1:400, respectively, of a hematoxylin and eosin stained acellular matrix prepared according to the present invention, demonstrating no cellular residues on the matrix.

FIG. 2 is a scanning electron micrograph of a diagonal portion of an acellular matrix prepared according to the present invention, demonstrating no cellular remnants in the matrix (magnification - 1:1000).

FIG. 3 is a scanning electron microscopy of a luminal side of an acellular matrix prepared according to the present invention demonstrating no cellular remnants on the matrix (1:100).

FIGs. 4a-b are light microscope photographs, magnified 1:400 and 1:100, respectively showing movat staining for collagen and elastin, demonstrating a fine layer of elastin in the luminal side of an acellular matrix and scarced throughout the outer side of the matrix, and the presence of collagen throughout the matrix.

FIG. 5 is a light microscope photograph demonstrating a confluent layer of human saphenous vein endothelial cells grown in culture having typical endothelial cell morphology (1:400).

FIG. 6 is a scanning electron micrograph demonstrating a confluent layer of human saphenous vein endothelial cells seeded on an acellular matrix of the present invention (1:100).

FIG. 7 is a photograph of an ethidium bromide stained agarose gel showing PCR products derived from a reverse transcribed NP-1 mRNA

extracted from samples as follows: C – HSVEC grown in culture; C/Mx – HSVEC grown on matrix; Mx – acellular matrix; H<sub>2</sub>O – water.

### DESCRIPTION OF THE PREFERRED EMBODIMENTS

5       The present invention is of a novel method of decellularizing biological matrices to thereby obtain acellular biological matrices of superior properties, which are used for the preparation of superior artificial organ/tissue implants, which are efficiently usable in corrective medicine. Specifically, the present invention can be used to replace, augment and/or  
10   repair hollow or planar malfunctioning, injured, diseased or absent tissues or organs, or portions thereof, in a subject in need thereof.

The principles and operation of according to the present invention may be better understood with reference to the drawings and accompanying descriptions.

15       Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it  
20   is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

According to one aspect of the present invention there is provided a method of decellularizing a biological matrix, so as to obtain an acellular biological matrix. The method according to this aspect of the present  
25   invention is effected by implementing the following method steps, in which, in a first step, a tissue of interest is obtained.

Obtaining the tissue of interest can be from any animal source, including, but not limited to, human beings and other mammals. It will be appreciated be one ordinarily skilled in the art that a true acellular  
30   biological matrix has little or no immunogenicity when implanted because

it is made mostly of collagen and elastin which are well known for low immunogenicity. For use in human beings it is presently preferred that the source of the tissue will be from an animal whose collagen and elastin amino acid sequences are highly similar or preferably identical to those of human beings, such as pigs and certain primates and monkeys. Yet, other sources are also applicable. The tissue of interest is harvested from its source by conventional. If the tissue of interest is harvested from a human being it can be of an autologous or heterologous source. In the latter case, the tissue of interest can be from a live or cadaveric donor. If autologous or from a live donor, the tissue of interest should be expendable. Animals, on the other hand can be sacrificed. The tissue of interest can be a two-dimensional organ/tissue of an animal or a portion thereof. By this it is meant that the tissue can be of a planar or hollow (bladder-like or tubular) structure. Tissues of planar nature include skin which is of exoderm origin and various endodermal and/or mesodermal membranes, such as the diaphragm. Tissues of tubular nature include all ducts, e.g., intestine, colon, vessels, e.g., blood vessels, urethras and ureters. Tissues of bladder-like nature include the bladder, stomach and the like. Alternatively, the tissue of interest can be a three-dimensional organ/tissue of an animal or a portion thereof. By this it is meant that tissue can be of substantial volume.

Thus, in one embodiment, the tissue of interest is derived from a gastrointestinal tract of an animal. It can therefore be the neo-esophagus, neo-stomach, small bowel, large bowel, biliary tract, a pancreatic duct, a rectum or portions thereof. In another embodiment, the tissue of interest is derived from a cardiovascular system of an animal. It can therefore be an artery, a vein, a heart valve, a heart wall or portions thereof. In yet another embodiment the tissue of interest is derived from a genitourinary system of an animal. It can therefore be a ureter, a kidney, a renal collecting system, a bladder, a urethra, a testis, a testis sac, a penis or portions thereof such as

corpus cavernosum. In still another embodiment the tissue of interest is derived from a skin or epidermis of an animal. In another embodiment the tissue of interest is derived from a parenchymal structure of an animal. It can therefore be a kidney, a liver or portions thereof. In yet another embodiment the tissue of interest is derived from a muscle structure of an animal. It can therefore be a heart muscle, a smooth muscle, a skeletal muscle or portions thereof. In still another embodiment the tissue of interest is derived from a skeletal structure of an animal. It can therefore be a cartilage, a tendon, a bone or portions thereof. In another embodiment the tissue of interest is derived from a nervous system of an animal. It can therefore be the dura or a nerve (e.g., a nerve bundle) or portions thereof.

Once the tissue of interest is obtained it is preferably first treated under hypotonic conditions sufficient to lyse red blood cells, yet, which maintain extracellular matrix architecture substantially intact. For a two-dimensional tissue, such conditions are fulfilled by immersion in tap or distilled water at 2 - 42 °C for less than 2 hours, preferably about 4 °C, preferably less than, or about, one hour. Overexposure of a tissue to hypotonic conditions may cause bloating and destruction of the collagen and elastin structures organization of the extracellular matrix thereof. Therefore, for most applications, in subsequent steps of the method of the present invention isotonic conditions are employed.

Following the mild hypotonic treatment as herein described, the tissue of interest is washed, as is further exemplified by the Examples that follow, so as to remove lysed red blood cells and it is then treated with a decellularization solution containing a cell lysis reagent. The cell lysis reagent preferably includes at least one non-ionic detergent, such as, but not limited to, TRITON-X 100, TWEEN 20, TWEEN 40, TWEEN 80, tetraethylene glycol monooctyl ether, heptaethylene glycol monododecyl ether, sucrose monolaurate, polyoxyethylene 20 cetyl ether (Brij 58),

polyoxyethylene 23 lauryl ether (Brij 35), octanoyl-N-methylglucamide (MEGA-8), N-octyl beta-D-glucopyranoside (OGP), lauryl maltoside (DDM), and PEG 600, and at least one mild base, such as, but not limited to a mild hydroxide base and a mild non-hydroxide base. By mild base it is meant a base that increases the pH of neutral water (pH 7.0) by no more than 2.0 pH units, preferably no more than 2.8 pH units. Mild hydroxide bases which can be used for implementing the method of the present invention include, without limitation, ammonium hydroxide, trimethylammonium hydroxide, triethanolammonium hydroxide, monoethanolammonium hydroxide, and benzylammonium hydroxide. Mild non-hydroxide bases include, without limitation, sodium acetate, sodium benzoate, sodium propionate and sodium phenoxide. According to presently preferred embodiments of the invention the non-ionic detergent is used in concentrations from 0.5 - 2.5 volume %, whereas the mild base is used in concentrations ranging from 0.5 - 2.0 M. Best results were so far achieved using the non-ionic detergent TRITON X100 in concentrations ranging from about 1 to about 1.5 volume % and the mild hydroxide base ammonium hydroxide a final concentration of about 10-100 mM, preferably about 50 mM. As used herein "about" means  $\pm 25$  %. Treating the tissue of interest with the decellularization solution is preferably accompanied by shaking, at 2-42 °C, preferably at about 4 °C, until a glistening white appearance of collagen is apparent and a sponge-like texture is obtained, typically following at least 24 hours optimally within about 48 hours for thin two-dimensional tissues and longer times for three dimensional tissues.

Following decellularization as described above cell remnants, as well as any residual ingredients of the decellularization solution are removed by washing, which is preferably effected under substantially isotonic conditions, e.g., in saline, typically at 2-42 °C, preferably at about

4 °C, in a shaker. The washing step is preferably repeated several times with a fresh isotonic solution. Alternating decellularization/washing steps may be advantageous for some applications. From one and up to 20 cycles or more of decellularization/washing steps may be employed for various applications.

It is believed, without limitation, that decellularization in isotonic conditions assist in preserving the macro and micro structure of the matrix as opposed to running tap water or distilled water, which cause the destruction of the matrix due to bloating.

Without limitation it is advisable that for most applications the use of harsh reagents and conditions such as trypsin and other proteolytic enzymes, SDS and other strong detergents and/or cross-linking agents, temperatures exceeding 50 °C, too vigorous/ruff shaking, and the like should be limited or avoided altogether. Trypsin, for example, is widely used for many indications. In tissue engineering, it is commonly used to separate confluent layers of cells by breaking the tight junctions between cells. It was experimentally found that excessive exposure to trypsin resulted in loss of shape and formation of irregular structures having regions of lower resistance to tensile stress. Aneurismatic areas were indeed observed in trypsin treated structures, especially when exposed to increasing pressures. The use of SDS is advisably avoided when practicing the present invention because even after excessive washes, residues of SDS still remain in the matrices. This was in particular evident in three-dimensional matrices. In addition, tissues treated with SDS significantly shrink and to a large extent loose their elastic properties. Furthermore, the shrinkage tends not to be uniform throughout the acellular matrix, resulting in an alteration in the three-dimensional structure of the extracellular matrix and a decrease in the luminal diameter thereof as was experimentally found for blood vessels, heart valve orifices

and ureters. Heart valve leaflets shrank as well under SDS treatment. As a result, blood vessels and ureters were much harder to handle, and heart valve leaflets suffered from a combined stenosis and insufficiency. Cross-linking agents (chemical reagents or physical agents such as ultraviolet (UV) radiation) are advantageously avoided as they reduce the cell adhesiveness properties of the resulting matrices.

Nevertheless, it is possible that for some applications the use, under controlled conditions of time and concentration, of harsh decellularization reagents may be advantageous.

Following a final wash, after which the acellular biological matrix include substantially no cells, cell remnants or residual decellularization agents, and according to a preferred embodiment of the present invention, the acellular biological matrix is dehydrated. Dehydration can be effected according to the present invention by baking, at, for example, 30 - 60 °C, preferably 40 - 50 °C, advantageously under reduced pressure, for several hours to several days. Preferably, dehydration is effected by freeze-drying (lyophilization) for several hours to several days or a few weeks. Typically, small two-dimensional structures require 26-30 hours of lyophilization. Three dimensional acellular biological matrices, which are derived as herein described from three dimensional tissues or portions thereof are preferably dehydrated as such. However, tubular, two-dimensional acellular biological matrices, which are derived, as herein described, from tubular, two dimensional tissues or portions thereof are preferably first cannulated over a cylindrical element, such as a silicon or TEFLON tube and are only then dehydrated. The cylindrical element is preferably selected so as to have a smooth surface. As a result, the lumen of dehydrated tubular matrices is characterized by smooth inner surface, which characteristic is maintained also after rehydration and is of immense importance when such matrices are used to construct artificial blood



vessels, urethras or ureters. It will be appreciated in this respect that lumen irregularity may result in undesired turbulent flow, which may lead to thrombosis in artificial blood vessels or to infection and stone formation in ureters and urethras. Similarly, bladder-like, two-dimensional acellular biological matrices, which are derived, as herein described, from bladder-like, two dimensional tissues, such as the urinary bladder or stomach are preferably dehydrated over a ball-like element, such as an inflatable balloon. The balloon is selected such that it will reach a desired volume under the under-pressure conditions employed in the dehydration process to thereby eliminate the possibility of non-uniform shrinkage of the during, for example, a freeze-drying process. Another option is to attach to a two-dimensional sheet-like structure, such as decellularized skin, aluminum foil or another form of a smooth sheet, to avoid non-uniform shrinkage of the matrix while dehydrated.

To a great extent the thickness of tissue dictates optimal decellularization, washing and dehydration conditions. The thicker the tissue is, longer incubation times, higher concentration of decellularization reagents, and longer dehydration periods are required. One of ordinary skills in the art would be able to optimize these parameters for any specific application.

Following dehydration, the acellular biological matrix is sterilized preferably by gas sterilization and is ready for use. Matrices as herein described maintain the extracellular matrix collagen/elastin ratio characteristic of the tissue for which they were derived. In addition, they maintain the fine micro-architecture of the extracellular matrix of the original tissue. Matrices prepared as described herein may be directly, preferably following rehydration, e.g., by saline, various conditioned media or serum, and optionally the addition thereto, e.g., by soaking, of one or more of a variety of growth factors, such as, but not limited to, VEGF and it's different isoforms, Basic FGF, EGF, PDGF, TGF-beta and

Angiopoietins and their isoforms, used as artificial tissues for transplantation, wherein infiltration of cells, colonization thereof and, in three-dimensional structures, also vascularization, will result in the transformation of the matrix into a functional artificial tissue *in-vivo*.

5 Systemic or local treatments can be administered so as to enhance the formation of functional artificial tissues. In addition, *in-vivo* seeding of suitable cell types may also be employed so as to enhance colonization.

Alternatively, a matrix as described herein can be used for the preparation of an artificial tissue/organ *in-vitro*, which artificial  
10 tissue/organ is thereafter implanted in a recipient in need thereof.

Thus, according to yet another aspect of the present invention there is provided a method of preparing an artificial implant for replacing, augmenting, bypassing or repairing a malfunctioning, injured, diseased or absent tissue, organ or a portion thereof, in a subject. The method  
15 according to this aspect of the present invention is effected by obtaining an acellular biological matrix using any one or combination of the embodiments of the method described herein above followed by seeding at least one cell type of interest *in-vitro* on the acellular biological matrix. While the source of the biological matrix employed is of lesser  
20 importance, it is presently preferred that the cell(s) seeded therein will be autologous, so as to prevent any type of immune response. Depending on the application, any cell type which can be cultured *in-vitro*, preferably as a primary culture, can be used for preparing artificial tissues in accordance with the teachings of the present invention. Thus, cell types that can be  
25 used include, but are not limited to, endothelial cells originating from arteries, veins or capillaries; fibroblast cells; dermal cells, including, but not limited to, keratinocytes, squamous cells, basal cells, melanocytes; different types of epithelial cells, such as urothelial cells, oral bucal cells, tracheal broncheal cells; hormone-endocrine secreting cells; hematopoietic  
30 stem cells (either from bone marrow or circulating), embryonal stem cells;

gastrointestinal mucosal cells, including without limitation, parietal cells, mucous cells, chief cells and enteroendocrine cells; smooth muscle cells, cardiac muscle cells, skeletal muscle cells; nervous system cells, including glial cells, satellite cells, astrocytes, oligodendrocytes; bone cells, including osteoblasts, osteocytes, osteoclasts, chondrocytes, hepatic cells including kupffer cells, endothelial cells, fat storing cells, sinusoidal capillary cells, renal cells, including renal derived epithelial, stromal and endothelial cells and hair follicle cells.

Harvesting and culturing these cell types are described in, for example, the text book "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition.

In a particular embodiment, the present invention is used to generate artificial blood vessels (also referred to herein as vascular constructs), in particular small-caliber blood vessels, which can be grafted to replace or bypass damaged blood vessels *in-vivo*. In another particular embodiment, the present invention is used to generate artificial urethras or ureters (also referred to herein as urinary constructs), which can be grafted to correct urinary system defects/malfunction. Acellular constructs prepared in accordance with the teachings of the present invention are not limited to organ-specific implantation. Seeded decellularized constructs can be implanted *in-vivo*, *ex-situ*. For example, a decellularized vein containing it's original one-way valve, seeded with urothelial cells can be implanted in a distal ureter and serve as an implanted anti-reflux mechanism.

Novel approaches for producing small-caliber arterial grafts have been extensively explored in recent years [Niklason et al., Science 284: 489-493, 1999; Huynh et al., Nature Biotech 17: 1083-1086, 1999; L'Heureux et al., Faseb J 12: 56, 1998] However, problems with mechanical properties, utilization of cells and occlusion have prevented a clinical breakthrough. Synthetic materials, when used to bypass arteries

less than 6 mm in diameter, have a thrombosis rate of over 40 % within 6 months post grafting [Sayers et al., Br. J Surg 85: 934, 1998]. Constructing a blood vessel by seeding cells on biodegradable polymer scaffolds, such as PGA, demonstrated an inflammatory response in the vessel wall and partial occlusion of the lumen was observed after as little as 26 days [Niklasson et al., Science 284: 489-493, 1999].

Badylak *et al.* was the first to introduce the concept of using small intestinal submucosa as a vascular graft [Badylak et al., J Surg Res 47: 74-80, 1989]. Huynh *et al.* introduced recently an improved model of utilizing an acellular collagen matrix as a small caliber graft [Huynh et al., [Huynh et al., Nature Biotech 17: 1083-1086, 1999]. They impregnated a collagen layer derived from small intestinal submucosa with a thin layer of dense fibrillar bovine collagen, cross-linked the structure with 1 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and coated it with heparin-benzalkonium chloride complex. This graft was implanted in the carotid position of a rabbit model with patency of up to 90 days. Their analysis showed that the grafts were endothelialized within 3 months, which contributed to their long-term patency. However, despite rapid surface coverage seen with animal models, trans-anastomotic endothelialization in humans has been limited, questioning the clinical applicability of this model [Davids et al., in: Tissue engineering of prosthetic vascular grafts 9 ed. Zilla & Griesler) 3-45. (R.G. Landes Co. Austin Texas; 1999; Berger et al., Ann Surg 175: 118-127, 1972; Sauvage et al., Arch Surg 109: 698-705, 1974].

The acellular matrices described herein provide readily available constructs that mimic native collagen and elastin content of a vessel, while ensuring mechanical properties comparable to pressure and mechanical stress that native blood vessels and grafted veins withstand in bypass surgery. The utilization of acellular matrices as vascular grafts is not new. The most reported in the literature is the small intestine submucosa, mainly

as a large diameter vascular graft [Badylak et al., J Surg Res 47: 74-80, 1989; Lantz et al., J Surg Res 53: 175-181, 1992; Huynh et al., Nature Biotech 17: 1083-1086, 1999]. An attempt to implant decellularized blood vessels without endothelial cells in dogs, was reported with limited success, mainly due to early occlusion in some of the grafts [Wilson et al., Ann Thorac Surg 60(2 Suppl):S353-358, 1995]. Although allogenic and xenogenic implants of collagen and collagen derivatives are known not to cause significant immunologic response [DeLustro et al., Clin Orthop 260: 263-279, 1990], Wilson et al. presented an encouraging finding that 6 months post grafting, the acellular matrices showed no inflammation and no leakage through the matrix wall [Wilson et al., Ann Thorac Surg 60(2 Suppl):S353-358, 1995]. These matrices, whether as xenografts from porcine origin or allografts from potential cadaveric donors may serve as an off-the-shelf substances for constructing tissue engineered blood vessels. In the treatment of a human, it would probably necessitate seeding autologous endothelial cells and achieving a confluent layer of endothelial cells prior to implantation.

HSVEC provide a readily available source of endothelial cells which can be harvested by a short out-patient procedure. HSVEC were not extensively studied in the past as a source of endothelial cells for tissue engineering of vascular constructs. As is further exemplified in the Examples section that follows, ten to fourteen days were required from cell harvesting and expansion *in-vitro*, until a critical amount of cells required to seed a 5 cm long acellular matrix was obtained (approximately  $5 \times 10^6$ ). It was further demonstrated that HSVEC maintain their phenotype and express normal endothelial cell markers up to 12 cell passages after cell expansion *in-vitro*, and are producing prostaglandin  $Fl\alpha$  and nitric oxide (NO), two potent vasodilators that are important for the preservation of graft patency [Babbette et al., Proc Natl. Acad Sci 74:

3922-3926, 1976; Shapira et al., Circulation 100(suppl II): II-322. - II-327, 1999].

There is an increasing amount of evidence of the importance of NO in vascular hemostasis [Keaney et al., Prog Cardiovas Dis 38: 129-154, 1995]. NO contributes to resting vascular tone [Quyyumi et al., J Clin Invest 95: 1747-1755, 1995], impairs platelet activation [Azuma et al., Br J Pharmacol 88: 411-415, 1986; Radomski et al., Biochem Biophys Res Commun 148: 1482-1489, 1989], and prevents leukocyte adhesion to the endothelium [Kubes et al., Am J Physiol 267: H931-H937, 1994]. These effects of NO on the vessel wall are important to protect the implanted graft against early thrombosis and later atherosclerosis [Keaney et al., Prog Cardiovasc Dis 38: 129-154, 1995]. Furthermore, the amount of NO production by seeded HSVEC on the acellular matrix, as determined by a relaxation effect thereof on aorta rings, was comparable to NO production from native radial and internal mammary artery endothelium as was described in other studies [Shapira et al., Circulation 100(suppl II): II-322 - II-327, 1999].

It is shown in the Examples section that follows that a confluent layer of HSVEC on the luminal side of an acellular matrix can be achieved within 3 to 5 days post seeding of about  $10^6$  cells per cm length. Previous studies utilizing collagen-based biologically derived matrices demonstrated adequate mechanical properties of blood vessel constructs. However, they failed to demonstrate long-term patency. This was due, at least in part, to the lack of a functioning endothelium. The HSVEC express normal endothelial cell markers and excrete vasoactive agents that enhance blood vessel patency. The study presented herein reveals that a blood vessel derived matrix, whether an allograft or a xenograft, in combination with a feasible source of endothelial cells is of clinical value for the replacement or bypass of diseased blood vessels in the human body.

During the dehydration procedure described herein water is removed from the acellular matrix, causing shrinkage of the matrix. Cannulating hollow structures such as blood vessels, ducts, ureters or urethras with very smooth silicone catheters enabled the matrix structures to remodel and adhere to the catheter and take the shape and smoothness of the silicon catheter. Therefore, the luminal side of these matrices is not thrombogenic in the blood system and fails to serve as a nidus for infection or stone formation in the urogenital system.

The dehydration procedure also increases the density and tensile strength of the acellular matrix, which is advantageous for structures that require high resistance to tensile strength, such as blood vessels and heart valves, and/or durability, such as cartilage structures in weight bearing joints. The major component of extracellular matrices and therefore of the acellular matrices is collagen. Since collagen implants tend to degrade through a sequential attack of lysosomal enzymes, *in-vivo* resorption of collagen matrices is regulated by the density of the implant. Thus, density is of more importance in structures that require longer time periods for cell infiltration, colonization and confluence, as such structures are subject to prolonged exposure to degradation processes *in-vivo*. Recent studies have demonstrated that the patency rate of tissue engineered blood vessels utilizing Polyglycolic Acid matrix seeded with cells do not exceed 4 weeks *in-vivo* [Niklasson *et al.*, Science 284:489-493, 1999]. In sharp distinction, blood vessel derived acellular matrices prepared in accordance with the teachings of the present invention were patent after 120 days in a sheep model without pre-seeding the matrices with endothelial cells.

As opposed to other mammals, e.g., sheep or dog, humans fail to efficiently repopulate vascular grafts with endothelial cells. Therefore, a clinical implant utilizing an acellular matrix is superior when preseeded with autologous human endothelial cells. Although previous attempts have been made to seed various autologous cells on acellular matrices,

lack of adhesion of these cells to the matrix was reported in most cases, including epithelial urothelial cells and endothelial cells [Sievert and Tanagho, World J. Urol. 18:19-25, 2000]. In sharp distinction, matrices prepared in accordance with the teachings of the present invention serve as  
5 excellent substances for cell adherence, resulting in confluent layers thereon of endothelial cells, epithelial urothelial cells, chondrocytes and smooth muscle cells.

Applications of the acellular matrices of the present invention include replacing, augmenting, bypassing or repairing a malfunctioning,  
10 injured, diseased or absent tissue, organ or a portion thereof in a subject in need thereof. In particular, the matrices of the present invention, *per se*, or after seeding with appropriate cells can be used to replace any hollow viscus within the human body. In the gastrointestinal tract, the neo-esophagus, neo-stomach, small and large bowels, biliary tract and  
15 pancreatic duct can be replaced. In the cardiovascular system, arteries and veins and heart valves can be replaced, repaired or bypassed and ventriculo-septal defect or atrial-septal defect can be repaired by suitable patches. In the genitourinary tract, patches can be prepared for the collecting system of the kidney, the ureter can be reconstructed, patches  
20 for bladder augmentation can be prepared, total bladder can be replaced, one-way-valves for vesico-ureteral reflux can be prepared and implanted, the urethra can be repaired or replaced, slings can be prepared for treating male and female urinary incontinence, ureteral and urethral stents for ureteral and urethral strictures can be prepared, replacement of testis after  
25 orchiectomy can be achieved and patches for the correction of various newborn anomalies such as hypospadias, bladder extrophy, vaginal and rectal agenesis can be prepared. Skin or dermal grafts can be prepared. Ear, Nose and Throat applications are also envisaged. Kidney and liver, muscular structures, including the heart muscle, smooth muscles and



skeletal muscles, as well as skeletal structures, including cartilage, tendons and bones can be augmented, replaced or repaired.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon  
5 examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated herein above and as claimed in the claims section below finds experimental support in the following examples.

### EXAMPLES

10 Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

### EXPERIMENTAL METHODS

#### *Decellularization protocol:*

15 Domesticated pigs weighting 20 to 30 Kg were obtained from a local breeder (Paterson Farm, MA). All donors were screened for human and animal viruses and were rejected if positive. Iliac, femoral arteries and ureters were excised, anastomoses were tied with 2/0 silk sutures and stored in saline for transportation to the experimentation facility. Blood  
20 vessels with an internal luminal size of 3 to 4 mm were cut into portions of approximately 4 cm in length. Ureters were similarly cut into portions of approximately 4 cm in length. Tissues were placed in distilled water in a mechanical shaker at 4 °C for one hour. Distilled water causes the rapid lysis of red blood cells. Tissues were thoroughly washed in running 0.9 %  
25 NaCl solution (saline) for 20 minutes to remove lysed red blood cells. Tissues were then placed in an isotonic/hypertonic decellularization solution containing TRITON X100 (1 % in volume for blood vessels; 1.5 % for ureters) and a final concentration of 10-100 mM, preferably about 50 mM, ammonium hydroxide in saline, and placed in a mechanical shaker  
30 (120 revolutions per minute) at 4 °C for 48 hours.

Thereafter, tissues were washed thoroughly with saline (0.9 % NaCl) to remove cellular remnants and the decellularization solution was replaced to a fresh one. The type and thickness of the tissue determine the number of decellularization/wash cycles, wherein thinner tissues require fewer cycles for completion of the decellularization process. In any case, tissues were considered macroscopically acellularized when a glistening white appearance of collagen became apparent and a sponge-like texture was obtained. This was also confirmed by other techniques, which are further described herein below. Acellular matrices were washed thoroughly with saline solution in a mechanical shaker at 4 °C for 24 hours. The saline solution was replaced every 4 hours. This assured the removal of detergent remnants from the matrices. After finalizing the decellularization process, matrices were cannulated with silicon catheters in order to allow shrinkage over the smooth surface of the silicon catheters. Thereafter, acellular matrices were lyophilized. Time of lyophilization was determined according to the type and thickness of the acellular matrix, whereas thicker matrices required longer lyophilization. Following lyophilization, the silicon catheters were removed and the acellular matrices were sterilized in cold gas.

***Saphenous endothelial cells (HSVEC) harvesting and isolation:***

Discarded segments of saphenous vein of patients undergoing coronary artery bypass surgery were placed in Endothelial Basal Medium (EBM) containing 20 % Fetal Bovine Serum (FBS). Segments of approximately 1 cm long were clamped at both ends and filled with 1 mg/ml type II collagenase (Sigma St. Louis, MO) in EBM and placed in a 5 % CO<sub>2</sub> humidified incubator for 20 minutes at 37 °C. The vein was then flushed with 50 ml of EBM and cells were collected by centrifugation for 10 minutes at 300 x g. Thereafter, cells were resuspended in 3 ml EBM containing 20 % FBS, 2 ng/ml of bFGF and 500 units/ml penicillin, and

placed onto small culture plates (3 cm in diameter) precoated with gelatin. After reaching near confluence, HSVEC were trypsinized and re-seeded on gelatin precoated large culture plates (10 cm in diameter). The medium was replaced and bFGF was added every other day.

5 During the first passage of the cells, some non-endothelial cells were present in the culture. In order to obtain a pure endothelial cell population, HSVEC were purified by immuno-isolation using Ulex europeaus I lectin (UEA-I, Vector, Burlingame, CA) coated magnetic particles concentration beads (Dynal, Norway) as previously described [10 Kraling et al., *In Vitro Cell Dev Biol Anim* 34:308-315, 1998]. Cell harvesting, immuno-isolation and re-seeding were repeated several times. In general, HSVEC went up to 12 passages before seeded onto an acellular matrix.

***Cell seeding on acellular biological matrix:***

15 A suspension of cultured HSVEC ( $5 \times 10^6$  cells/ml) was injected into decellularized blood vessels prepared as described above. The cell containing vessels were placed in a humidified incubator and frequently rotated for one hour. Thereafter, EBM containing 20 % FBS and 2 ng/ml bFGF was gently added. The medium was replaced every 2-3 days. Cells 20 were allowed to grow for 5 to 7 days to reach confluency on the inner walls of the matrix.

***Mechanical strength - burst pressure***

Native pig blood vessels, acellular matrices and matrices pre-seeded with HSVEC that reached confluency were cannulated on a specially 25 designed system as previously described [L'Heureux et al., *Faseb J* 12: 56, 1998] and were pressurized with phosphate buffered saline (Sigma). All vessels were 5 cm in length and of an internal diameter of approximately 4 mm. Hydrostatic pressure was increased slowly up to 900 mmHg.

### *Synthesis of prostaglandin F1 $\alpha$*

Synthesis of prostaglandin F1 $\alpha$  was determined by measuring 6-keto prostaglandin F1 $\alpha$  using an enzyme immunoassay kit according to the manufacturer's instructions (Cayman Chemical, Cayman, Islands).  
5 Briefly, 0.1 ml of condition medium was diluted in acetic acid and a series of dilutions were assayed in order to achieve a log of volume-dependent concentrations curve. Results were measured according to the plotted volume-concentration curve and prostaglandin F1 $\alpha$  levels were calculated in the condition medium per 1 cm<sup>2</sup> of confluent cells on a culture dish or  
10 on 1 cm<sup>2</sup> of confluent cells seeded on a matrix.

### *Nitric oxide (NO) production:*

NO mediated vascular relaxation was evaluated in an organ-chamber. Guinea pig thoracic aorta was harvested, endothelium was removed by gentle rubbing and the aorta was cut into 5 mm rings.  
15 Each ring was suspended between 2 tungsten stirrups for measurement of isometric tension as previously described [Keaney Proc Natl Acad Sci USA 90: 11880-11884, 1993; Keaney et al., J Clin Invest 95: 2520-2529, 1995; Keaney et al., J Clin Invest 93: 844-851, 1994] and placed in an organ chamber containing 10 ml Kreb's buffer solution at 37 °C with an  
20 atmosphere of 5 % CO<sub>2</sub>, 15 % O<sub>2</sub> and 80 % N<sub>2</sub>. Each engineered cell-matrix construct (2-3 cm in length) was tied to a 21 Gauge needle, which was attached to a plastic intravenous tubing and placed above the organ chamber. The rings were contracted with 80 mM KCl Kreb's buffer in a stepwise fashion to obtain a resting tension of 4 grams. After resting  
25 for 90 minutes, rings were contracted with prostaglandin F2 $\alpha$  until a stable contraction of approximately 50 % of maximum KCl contraction was obtained (typically a concentration of 10<sup>-7</sup> M prostaglandin F2 $\alpha$  was required. Then, vasoactive agents were added with an infusion pump through the cell-matrix constructs for the estimation of basal NO

production as previously described [Rees et al., Br. J Pharmacol 101: 746-752, 1990; Moro et al., Proc Natl Acad Sci USA 93: 1480-1485, 1995]. Dose-response curves for all vasoactive agents were compared among groups by two-way ANOVA for repeated measurements.

5       ***Scanning electron microscopy:***

Native pig blood vessels, acellular matrices and matrices seeded with HSVEC were fixed in 1 % (v/v) buffered glutaraldehyde and 0.1% (v/v) buffered formaldehyde for 30 minutes and 24 hours, respectively, dehydrated through a graded ethanol series, and air dried. The dried  
10 samples were mounted on aluminum supports and sputter coated with gold. A scanning electron microscope (JOEL, model JSM-35, Peabody, MA) was operated to image specimens at 25-kV voltage.

***Histological and immunocytochemical analyses:***

Acellular matrices and matrices seeded with HSVEC were  
15 immersed in Ornitin Carbamoyl Transferase (OCT; Sakura Finetek; Torrance, CA) and frozen in liquid nitrogen. Cryostat microsections (5 µm in thickness) were prepared and analyzed histologically after staining with hematoxylin and eosin or movat staining [Garvey et al., Stain Technol 61: 60-62, 1986], and immunohistochemically using anti-CD-31  
20 (Faminogen; Rockville, MA), anti-Factor 8 (Daco Laboratories), anti-KDR (Santa Cruz; Santa Cruz, CA), anti-FLT (Santa Cruz; Santa Cruz, CA), anti-NP1 (a gift from Alex Kolodkin, Johns Hopkins, Baltimore, MD) and anti-smooth muscle actin antibodies (Daco Laboratories). The primary antibodies were detected using the Vectra  
25 avidin-biotin-immunoperoxidase method according to the manufacturer's instructions. As a negative control, the primary antibody was omitted from the reaction.

***RNA isolation:***

Acellular matrices, acellular matrices seeded with HSVEC and  
30 plate grown HSVEC were homogenized in RNazol reagent at 40 °C using

a tissue homogenizer. RNA was isolated according to the manufacturers protocol (Tel-Test). Complementary DNA (cDNA) was synthesized from 2 µl RNA samples using the Superscript II reverse transcriptase (Gibco BRL) and random hexamers as primers.

5       ***RT-PCR:***

For PCR amplifications 2 µl cDNA sample, 1 U Taq DNA polymerase (Roche), 0.2 mM dNTPs and 30 pM of each amplification primer were mixed in a final volume of 30 µl. PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining.

10       PCR primers for amplification of NP-1 cDNA were:

5'-TTTCGCAACGATAAATGTGGCGAT-3' (SEQ ID NO:1) and  
5'-TATCACTCCACTAGGTGTTG-3' (SEQ ID NO:2).

***Western blotting:***

HSVEC cultured in culture plates (10 cm in diameter) were lysed in  
15 a buffer containing 10 mM Tris-HCl, pH 7.0, 50 mM NaCl, 1 % TRITON  
100X and protease inhibitor (Sigma). Anti-KDR and anti-NP1 (1:100  
dilution) antibodies were added to the cell lysate and samples were  
incubated at 4 °C for 4 – 18 hours. Protein G-sepharose was added, and  
samples were incubated 10 – 30 minutes at room temperature. Conjugates  
20 were spun down and pellets were washed in PBS. Pellets were  
resuspended in 25 µl 1x sample buffer with 1.2 % SDS. Samples were  
boiled for 5 minutes and loaded on a 6 % SDS-PAGE and electrophoresed  
for about 4 hours at a constant current of 25 mA. Proteins were blotted  
onto PVDF-membranes, the membranes were incubated with anti-NP1 and  
25 anti-KDR antibodies overnight. Subsequently, membranes were incubated  
with secondary antibodies for 30 minutes. The signal was visualized using  
the ECL system (New-England Nuclear).

## EXPERIMENTAL RESULTS

### *Acellular matrices:*

Vessels were placed in distilled water for one hour to induce red blood cell lysis, then thoroughly washed, and placed in a decellularization solution containing 1 % - 1.5 % TRITON 100X and 10-100 mM, preferably about 50 mM, ammonium hydroxide in saline and placed in a mechanical shaker (120 RPM) at 4 °C for 48 hours. Vessels were washed thoroughly and the decellularization solution was replaced. After finalizing the cleaning process, matrices were lyophilized (Virtis; Gardiner, NY) over a smooth silicone catheter and sterilized in cold gas. Following lyophylization, macroscopically, the acellular matrix appeared white and with a sponge-like texture. Hematoxylin and eosin staining (Figures 1a-b) and SEM analysis (Figures 2 and 3) of the acellular matrices demonstrated the absence of cellular residues.

It was experimentally found that the type and thickness of the tissue undergoing decellularization determines the optimal concentrations of TRITON X100 and ammonium hydroxide to be used. Most two-dimensional tissues, including fascia, nerves and blood vessels, required the above mentioned concentrations for optimal results. Parenchymal tissues, such as, but not limited to, required a higher concentration of TRITON X100 and 1 M ammonium hydroxide, e.g., 1.5-2 % in volume of TRITON X100 and about 100 mM ammonium hydroxide.

In order to confirm acellularity of the matrix, RNA detection was attempted. RNA was extracted using RNeasy and RT-PCR of the house keeping gene GAP-DH was thereafter attempted. PCR products were fractionated by gel electrophoresis, and gene expression was quantified using a measurement of band intensity or densitometry. RT-PCR performed on acellular matrices failed to amplify the GAP-DH gene, suggesting that no cellular moiety is present within the matrix (Figure 7).

Movat staining for collagen and elastin was used to demonstrate the presence of collagen and the preservation of a fine layer of elastin in the luminal side of the acellular matrix (Figures 4a-b). Taken together, these results suggest that this proposed cleaning procedure yields an acellular matrix, while preserving the extracellular layers of collagen and elastin.

***HSVEC isolation and characterization:***

Figure 5 demonstrates a confluent layer of the HSVEC with a typical endothelial cell morphology. Immunostaining with anti-CD-31 antibody and anti-Factor 8 antibody, two well characterized human endothelial cell markers, positively stained the HSVEC, while anti-smooth muscle actin antibody failed to demonstrate any contaminating muscle cells in the culture. To further confirm functionality of the cells, western blotting with anti-KDR and anti-NP1 antibodies, both markers for vascular endothelial growth factor (VEGF) receptor on endothelial cells, showed the presence of VEGF receptors on the cultured HSVEC.

***HSVEC seeded on the acellular matrix:***

Figure 6 demonstrates the development of a confluent layer of HSVEC on the matrix 5 days after seeding. Hematoxylin and eosin staining and positive immunostaining with anti-CD-31 antibody showed the presence of a fine layer of HSVEC on the matrix.

In an attempt to evaluate the viability of the HSVEC *in-vivo*, hematoxylin and eosin staining and positive immunostaining with anti CD-31 antibody confirmed the presence of the HSVEC on the matrix 7 days after implantation *in-vivo*, *ex-situ* in the subcutaneous space of athymic mice.

RT-PCR demonstrated the presence of NP1 receptor RNA in the HSVEC in culture and after seeding on the matrix (Figure 7).

***Functionality Studies:***

To evaluate the preservation of functionality characteristics of HSVEC seeded on acellular matrices an assay to evaluate the synthesis of



prostaglandin  $F1\alpha$ , a potent inhibitor of platelet aggregation and a vascular smooth muscle relaxant was performed [Babbette et al., Proc Natl Acad Sci USA 74: 3922-3926, 1977]. The mean levels (6 independent experiments) of prostaglandin  $F1\alpha$  secreted to the growth medium of 1 cm<sup>2</sup> confluent HSVEC in culture and 1 cm<sup>2</sup> confluent HSVEC grown on the inner walls of an acellularized matrix were 1.2 ng/ml and 0.9 ng/ml, respectively.

Calcium Ionophore A23, which enhances NO production by endothelial cells, was injected through the cell-matrix construct in increasing concentrations and relaxation of an aortic ring was measured. Thereafter, calcium ionophore A23 was injected with NG-nitro-L-arginine methyl ester (L-NAME) which blocks NO production. The contractile response to increasing concentrations of L-NAME was recorded as the percent of maximum contraction produced by 80 mM KCl [Shapira et al., Circulation 100(suppl II): II-322 - II-327, 1999]. As a control of relaxation activity of the aortic rings, SNP, a non-endothelial cells dependent smooth muscle relaxant was injected. Relaxation of aortic rings in response to A23 mediated NO secretion from HSVEC was up to 80 %.

***Mechanical properties of acellular matrices:***

Native pig blood vessels, acellular matrices and matrices pre-seeded with HSVEC that reached confluency were cannulated on a specially designed system as previously described [L'Heureux et al., Faseb J 12: 56, 1998] and were pressurized with phosphate buffered saline (Sigma). Hydrostatic pressure was increased slowly up to 900 mmHg. All samples withstood the pressure without breaking or leaking.

***Patency of acellular matrices:***

Decellularized blood vessel matrices prepared as described herein without pre-seeding the matrices with endothelial cells were patent after 120 days in a sheep model.

***Cell seeding on acellular matrices:***

Matrices of different tissue origin prepared as described herein served as appropriate substances for cell adherence, resulting in confluent layers of endothelial cells, epithelial urothelial cells, chondrocytes and smooth muscle cells.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

## WHAT IS CLAIMED IS:

1. A method of decellularizing a biological matrix so as to obtain an acellular biological matrix, the method comprising steps of:

- (a) obtaining a tissue of interest;
- (b) treating said tissue of interest with a decellularization solution containing at least one non-ionic detergent and at least one mild base; and
- (c) removing cell remnants, thereby obtaining the acellular biological matrix.

2. The method of claim 1, wherein prior to step (b) said tissue of interest is treated under hypotonic conditions sufficient to lyse red blood cells, yet which maintain extracellular matrix architecture.

3. The method of claim 2, wherein said hypotonic conditions include immersion in distilled water at 2-42 °C for less than 2 hours.

4. The method of claim 1, wherein said non-ionic detergent is selected from the group consisting of TRITON-X 100, TWEEN 20, TWEEN 40, TWEEN 80, tetraethylene glycol monoethyl ether, heptaethylene glycol monododecyl ether, sucrose monolaurate, polyoxyethylene 20 cetyl ether (Brij 58), polyoxyethylene 23 lauryl ether (Brij 35), octanoyl-N-methylglucamide (MEGA-8), N-octyl beta-D-glucopyranoside (OGP), lauryl maltoside (DDM) and PEG 600.

5. The method of claim 1, wherein said at least one mild base is selected from the group consisting of a mild hydroxide base and a mild non-hydroxide base.

6. The method of claim 1, wherein steps (b) and (c) are performed under substantially isotonic conditions.

7. The method of claim 1, wherein step (c) is effected by repeated washes with an isotonic solution.

8. The method of claim 1, wherein steps (b) and (c) are repeated in sequence at least twice.

9. The method of claim 1, wherein step (b) is accompanied by shaking.

10. The method of claim 1, wherein said non-ionic detergent is employed at a concentration of 0.5 - 2.5 volume %.

11. The method of claim 1, wherein said mild hydroxide base is employed at a final concentration of 10-100 mM.

12. The method of claim 1, wherein step (b) is performed at 2-42 °C.

13. The method of claim 1, wherein step (b) is performed for at least 24 hours.

14. The method of claim 1, wherein step (b) is performed for about 48 hours.

15. The method of claim 1, further comprising the step of dehydrating the acellular biological matrix.

16. The method of claim 15, wherein said step of dehydrating the acellular biological matrix is effected by baking.
17. The method of claim 15, wherein said step of dehydrating the acellular biological matrix is effected by freeze-drying.
18. The method of claim 15, wherein said step of dehydrating the acellular biological matrix is effected by lyophilization.
19. The method of claim 15, wherein said step of dehydrating the acellular biological matrix is effected at underpressure.
20. The method of claim 15, wherein said tissue is a tubular tissue, whereas prior to said step of dehydrating the acellular biological matrix, the acellular biological matrix is mounted over a cylindrical element.
21. The method of claim 20, further comprising the step of removing the acellular biological matrix from said cylindrical element.
22. The method of claim 20, wherein said cylindrical element is a silicon tube.
23. The method of claim 1, wherein step (b) is continued or repeated until a glistening white appearance of collagen is apparent and a sponge-like texture is obtained.
24. The method of claim 1, wherein said tissue of interest is derived from a gastrointestinal tract of an animal.

25. The method of claim 24, wherein said tissue is selected from the group consisting of neo-esophagus, neo-stomach, small bowel, large bowel, biliary tract, a pancreatic duct and a rectum.

26. The method of claim 1, wherein said tissue of interest is derived from a cardiovascular system of an animal.

27. The method of claim 26, wherein said tissue is selected from the group consisting of an artery, a vein, a heart valve and a heart wall.

28. The method of claim 1, wherein said tissue of interest is derived from a genitourinary system of an animal.

29. The method of claim 28, wherein said tissue is selected from the group consisting of a ureter, a kidney, a bladder, a urethra, renal collecting system, a testis, a testis sac and a penis.

30. The method of claim 1, wherein said tissue of interest is derived from a skin of an animal.

31. The method of claim 1, wherein said tissue of interest is derived from an epidermis of an animal.

32. The method of claim 1, wherein said tissue of interest is derived from an endodermis of an animal.

33. The method of claim 1, wherein said tissue of interest is derived from a mesoderm of an animal.

34. The method of claim 1, wherein said tissue of interest is derived from a parenchymal structure of an animal.

35. The method of claim 34, wherein said parenchymal structure is selected from the group consisting of kidney and liver.

36. The method of claim 1, wherein said tissue of interest is derived from a muscle structure of an animal.

38. The method of claim 36, wherein said muscle structure is selected from the group consisting of a heart muscle, a smooth muscle, and a skeletal muscle.

39. The method of claim 1, wherein said tissue of interest is derived from a skeletal structure of an animal.

40. The method of claim 39, wherein said skeletal structure is selected from the group consisting of a cartilage, a tendon and a bone.

41. The method of claim 1, wherein said tissue of interest is derived from a nervous system of an animal.

42. The method of claim 41, wherein said tissue is selected from the group consisting of a dura and a nerve.

43. The method of claim 1, wherein said tissue of interest is derived from a two-dimensional organ of an animal.

44. The method of claim 1, wherein said tissue of interest is derived from a three-dimensional organ of an animal.

45. The method of claim 1, wherein said tissue of interest is derived from a human being.

46. The method of claim 1, wherein said tissue of interest is derived from a mammal.

47. The method of claim 1, wherein said mammal is a pig.

48. An acellular matrix obtained by the method of claim 1.

49. An acellular matrix obtained by the method of claim 15.

50. A method of preparing an artificial implant for replacing, augmenting, bypassing or repairing a malfunctioning, injured, diseased or absent tissue, organ or a portion thereof, in a subject, the method comprising the steps of:

- (a) obtaining the acellular biological matrix of claim 48; and
- (b) seeding at least one cell type of interest on the acellular biological matrix.

51. The method of claim 50, wherein said at least one cell type of interest is autologous.

52. The method of claim 50, wherein said at least one cell type of interest is selected from the group consisting of endothelial cells, fibroblast cells, dermal cells, epithelial cells, hormone-endocrine secreting cells; hematopoietic stem cells, embryonal stem cells, gastrointestinal mucosal cells, smooth muscle cells, cardiac muscle cells, skeletal muscle cells, nervous system cells, bone cells, hepatic cells, fat storing cells, sinusoidal capillary cells, renal cells and hair follicle cells.



53. The method of claim 50, wherein the acellular biological matrix is derived from a two-dimensional tissue.

54. An artificial implant prepared using the method of claim 50.

55. A method of replacing, augmenting, bypassing or repairing a malfunctioning, injured, diseased or absent tissue, organ or a portion thereof, in a subject, the method comprising the steps of:

- (a) obtaining the artificial implant of claim 54; and
- (b) implanting the artificial implant in the subject.

56. The method of claim 55, wherein said at least one cell type of interest autologous.

57. The method of claim 55, wherein said at least one cell type of interest is selected from the group consisting of endothelial cells, fibroblast cells, dermal cells, epithelial cells, hormone-endocrine secreting cells; hematopoietic stem cells, embryonal stem cells, gastrointestinal mucosal cells, smooth muscle cells, cardiac muscle cells, skeletal muscle cells, nervous system cells, bone cells, hepatic cells, fat storing cells, sinusoidal capillary cells, renal cells and hair follicle cells.

58. The method of claim 55, wherein the acellular biological matrix is derived from a two-dimensional tissue.

59. A method of preparing an artificial implant for replacing, augmenting, bypassing or repairing a malfunctioning, injured, diseased or absent tissue, organ or a portion thereof, in a subject, the method comprising the steps of:

- (a) obtaining the acellular biological matrix of claim 49; and

- (b) seeding at least one cell type of interest on the acellular biological matrix.

60. The method of claim 59, wherein said at least one cell type of interest is autologous.

61. The method of claim 59, wherein said at least one cell type of interest is selected from the group consisting of endothelial cells, fibroblast cells, dermal cells, epithelial cells, hormone-endocrine secreting cells; hematopoietic stem cells, embryonal stem cells, gastrointestinal mucosal cells, smooth muscle cells, cardiac muscle cells, skeletal muscle cells, nervous system cells, bone cells, hepatic cells, fat storing cells, sinusoidal capillary cells, renal cells and hair follicle cells.

62. The method of claim 59, wherein the acellular biological matrix is derived from a two-dimensional tissue.

63. An artificial implant prepared using the method of claim 59.

64. A method of replacing, augmenting, bypassing or repairing a malfunctioning, injured, diseased or absent tissue, organ or a portion thereof, in a subject, the method comprising the steps of:

- (a) obtaining the artificial implant of claim 63; and
- (b) implanting the artificial implant in the subject.

65. The method of claim 64, wherein said at least one cell type of interest is autologous.

66. The method of claim 64, wherein said at least one cell type of interest is selected from the group consisting of endothelial cells,

fibroblast cells, dermal cells, epithelial cells, hormone-endocrine secreting cells; hematopoietic stem cells, embryonal stem cells, gastrointestinal mucosal cells, smooth muscle cells, cardiac muscle cells, skeletal muscle cells, nervous system cells, bone cells, hepatic cells, fat storing cells, sinusoidal capillary cells, renal cells and hair follicle cells. 67. The method of claim 64, wherein the acellular biological matrix is derived from a two-dimensional tissue:

68. A method of replacing, augmenting, bypassing or repairing a malfunctioning, injured, diseased or absent tissue, organ or a portion thereof, in a subject, the method comprising the steps of:

- (a) obtaining the acellular biological matrix of claim 48; and
- (b) implanting the acellular biological matrix in the subject.

69. A method of replacing, augmenting, bypassing or repairing a malfunctioning, injured, diseased or absent tissue, organ or a portion thereof, in a subject, the method comprising the steps of:

- (a) obtaining the acellular biological matrix of claim 49; and
- (b) implanting the acellular biological matrix in the subject.

70. A method of decellularizing a biological matrix so as to obtain an acellular biological matrix, the method comprising the steps of:

- (a) obtaining a tissue of interest;
- (b) treating the tissue of interest with a decellularization solution containing at least one cell lysing agent;
- (c) removing cell remnants; and
- (d) dehydrating, thereby obtaining the acellular biological matrix.

71. The method of claim 70, wherein prior to step (b) said tissue of interest is treated under hypotonic conditions sufficient to lyse red blood cells, yet which maintain extracellular matrix architecture.

72. The method of claim 71, wherein said hypotonic conditions include immersion in distilled water at 2-42 °C for less than 2 hours.

73. The method of claim 70, wherein steps (b) and (c) are performed under substantially isotonic conditions.

74. The method of claim 70, wherein step (c) is effected by repeated washes with an isotonic solution.

75. The method of claim 70, wherein steps (b) and (c) are repeated in sequence at least twice.

76. The method of claim 70, wherein step (b) is accompanied by shaking.

77. The method of claim 70, wherein step (b) is performed at 2-42 °C.

78. The method of claim 70, wherein step (b) is performed for at least 24 hours.

79. The method of claim 70, wherein step (b) is performed for about 48 hours.

80. The method of claim 70, wherein said step of dehydrating is effected by baking.

81. The method of claim 70, wherein said step of dehydrating is effected by freeze-drying.

82. The method of claim 70, wherein said step of dehydrating is effected by lyophilization.

83. The method of claim 70, wherein said step of dehydrating is effected at underpressure.

84. The method of claim 70, wherein said tissue is a tubular tissue, whereas prior to said step of dehydrating, the acellular biological matrix is mounted over a cylindrical element.

85. The method of claim 84, further comprising the step of removing the acellular biological matrix from said cylindrical element.

86. The method of claim 84, wherein said cylindrical element is a silicon tube.

87. The method of claim 20, wherein step (b) is continued or repeated until a glistening white appearance of collagen is apparent and a sponge-like texture is obtained.

88. The method of claim 20, wherein said at least one cell lysing agent is selected from the group consisting of a non-ionic detergent and a mild base.

89. The method of claim 88, wherein said non-ionic detergent is selected from the group consisting of TRITON-X 100, TWEEN 20, TWEEN 40, TWEEN 80, tetraethylene glycol mono-octyl ether, heptaethylene glycol monododecyl ether, sucrose monolaurate, polyoxyethylene 20 cetyl ether (Brij 58), polyoxyethylene 23 lauryl ether (Brij 35), octanoyl-N-methylglucamide (MEGA-8), N-octyl beta-D-glucopyranoside (OGP), lauryl maltoside (DDM), and PEG 600.

90. The method of claim 88, wherein said at least one mild base is selected from the group consisting said at least one mild base is selected from the group consisting of a mild hydroxide base and a mild non-hydroxide base.

91. The method of claim 88, wherein said non-ionic detergent is employed at a concentration of 0.5 - 2.5 volume %.

92. The method of claim 88, wherein said at least one mild base is employed at a final concentration of 10-100 mM.

93. The method of claim 70, wherein said tissue of interest is derived from a gastrointestinal tract of an animal.

94. The method of claim 93, wherein said tissue is selected from the group consisting of neo-esophagus, neo-stomach, small bowel, large bowel, biliary tract, a pancreatic duct and a rectum.

95. The method of claim 70, wherein said tissue of interest is derived from a cardiovascular system of an animal.

96. The method of claim 95, wherein said tissue is selected from the group consisting of an artery, a vein, a heart valve and a heart wall.

97. The method of claim 70, wherein said tissue of interest is derived from a genitourinary system of an animal.

98. The method of claim 97, wherein said tissue is selected from the group consisting of a ureter, a kidney, a bladder, a urethra, a renal collecting system, a testis, a testis sac and a penis.

99. The method of claim 70, wherein said tissue of interest is derived from a skin of an animal.

100. The method of claim 70, wherein said tissue of interest is derived from an epidermis of an animal.

101. The method of claim 70, wherein said tissue of interest is derived from an endodermis of an animal.

102. The method of claim 70, wherein said tissue of interest is derived from a mesoderm of an animal.

103. The method of claim 70, wherein said tissue of interest is derived from a parenchymal structure of an animal.

104. The method of claim 103, wherein said parenchymal structure is selected from the group consisting of kidney and liver.

105. The method of claim 70, wherein said tissue of interest is derived from a muscle structure of an animal.

106. The method of claim 105, wherein said muscle structure is selected from the group consisting of a heart muscle, a smooth muscle, and a skeletal muscle.

107. The method of claim 70, wherein said tissue of interest is derived from a skeletal structure of an animal.

108. The method of claim 107, wherein said skeletal structure is selected from the group consisting of a cartilage, a tendon and a bone.

109. The method of claim 70, wherein said tissue of interest is derived from a nervous system of an animal.

110. The method of claim 109, wherein said tissue is selected from the group consisting of a dura and a nerve.

111. The method of claim 70, wherein said tissue of interest is derived from a two-dimensional organ of an animal.

112. The method of claim 70, wherein said tissue of interest is derived from a three-dimensional organ of an animal.

113. The method of claim 70, wherein said tissue of interest is derived from a human being.

114. The method of claim 70, wherein said tissue of interest is derived from a mammal.

115. The method of claim 70, wherein said mammal is a pig.



116. An acellular matrix obtained by the method of claim 70.
117. An acellular matrix obtained by the method of claim 88.
118. A method of preparing an artificial implant for replacing, augmenting, bypassing or repairing a malfunctioning, injured, diseased or absent tissue, organ or a portion thereof, in a subject, the method comprising the steps of:
- (a) obtaining the acellular biological matrix of claim 116; and
  - (b) seeding at least one cell type of interest on the acellular biological matrix.
119. The method of claim 118, wherein said at least one cell type of interest is autologous.
120. The method of claim 118, wherein said at least one cell type of interest is selected from the group consisting of endothelial cells, fibroblast cells, dermal cells, epithelial cells, hormone-endocrine secreting cells; hematopoietic stem cells, embryonal stem cells, gastrointestinal mucosal cells, smooth muscle cells, cardiac muscle cells, skeletal muscle cells, nervous system cells, bone cells, hepatic cells, fat storing cells, sinusoidal capillary cells, renal cells and hair follicle cells.
121. The method of claim 118, wherein the acellular biological matrix is derived from a two-dimensional tissue.
122. An artificial implant prepared using the method of claim 118.

123. A method of replacing, augmenting, bypassing or repairing a malfunctioning, injured, diseased or absent tissue, organ or a portion thereof, in a subject, the method comprising the steps of:

- (a) obtaining the artificial implant of claim 122; and
- (b) implanting the artificial implant in the subject.

124. The method of claim 123, wherein said at least one cell type of interest is autologous.

125. The method of claim 123, wherein said at least one cell type of interest is selected from the group consisting of endothelial cells, fibroblast cells, dermal cells, epithelial cells, hormone-endocrine secreting cells; hematopoietic stem cells, embryonal stem cells, gastrointestinal mucosal cells, smooth muscle cells, cardiac muscle cells, skeletal muscle cells, nervous system cells, bone cells, hepatic cells, fat storing cells, sinusoidal capillary cells, renal cells and hair follicle cells.

126. The method of claim 123, wherein the acellular biological matrix is derived from a two-dimensional tissue.

127. A method of preparing an artificial implant for replacing, augmenting, bypassing or repairing a malfunctioning, injured, diseased or absent tissue, organ or a portion thereof, in a subject, the method comprising the steps of:

- (a) obtaining the acellular biological matrix of claim 117; and
- (b) seeding at least one cell type of interest on the acellular biological matrix.

128. The method of claim 127, wherein said at least one cell type of interest is autologous.

129. The method of claim 127, wherein said at least one cell type of interest is selected from the group consisting of endothelial cells, fibroblast cells, dermal cells, epithelial cells, hormone-endocrine secreting cells; hematopoietic stem cells, embryonal stem cells, gastrointestinal mucosal cells, smooth muscle cells, cardiac muscle cells, skeletal muscle cells, nervous system cells, bone cells, hepatic cells, fat storing cells, sinusoidal capillary cells, renal cells and hair follicle cells. 130. The method of claim 127, wherein the acellular biological matrix is derived from a two-dimensional tissue.

131. An artificial implant prepared using the method of claim 127.

132. A method of replacing, augmenting, bypassing or repairing a malfunctioning, injured, diseased or absent tissue, organ or a portion thereof, in a subject, the method comprising the steps of:

- (a) obtaining the artificial implant of claim 131; and
- (b) implanting the artificial implant in the subject.

133. The method of claim 132, wherein said at least one cell type of interest is autologous.

134. The method of claim 132, wherein said at least one cell type of interest is selected from the group consisting of endothelial cells, fibroblast cells, dermal cells, epithelial cells, hormone-endocrine secreting cells; hematopoietic stem cells, embryonal stem cells, gastrointestinal mucosal cells, smooth muscle cells, cardiac muscle cells, skeletal muscle cells, nervous system cells, bone cells, hepatic cells, fat storing cells, sinusoidal capillary cells, renal cells and hair follicle cells.

135. The method of claim 132, wherein the acellular biological matrix is derived from a two-dimensional tissue.

136. A method of replacing, augmenting, bypassing or repairing a malfunctioning, injured, diseased or absent tissue, organ or a portion thereof, in a subject, the method comprising the steps of:

- (a) obtaining the acellular biological matrix of claim 116; and
- (b) implanting the acellular biological matrix in the subject.

137. A method of replacing, augmenting, bypassing or repairing a malfunctioning, injured, diseased or absent tissue, organ or a portion thereof, in a subject, the method comprising the steps of:

- (a) obtaining the acellular biological matrix of claim 117; and
- (b) implanting the acellular biological matrix in the subject.

1/7

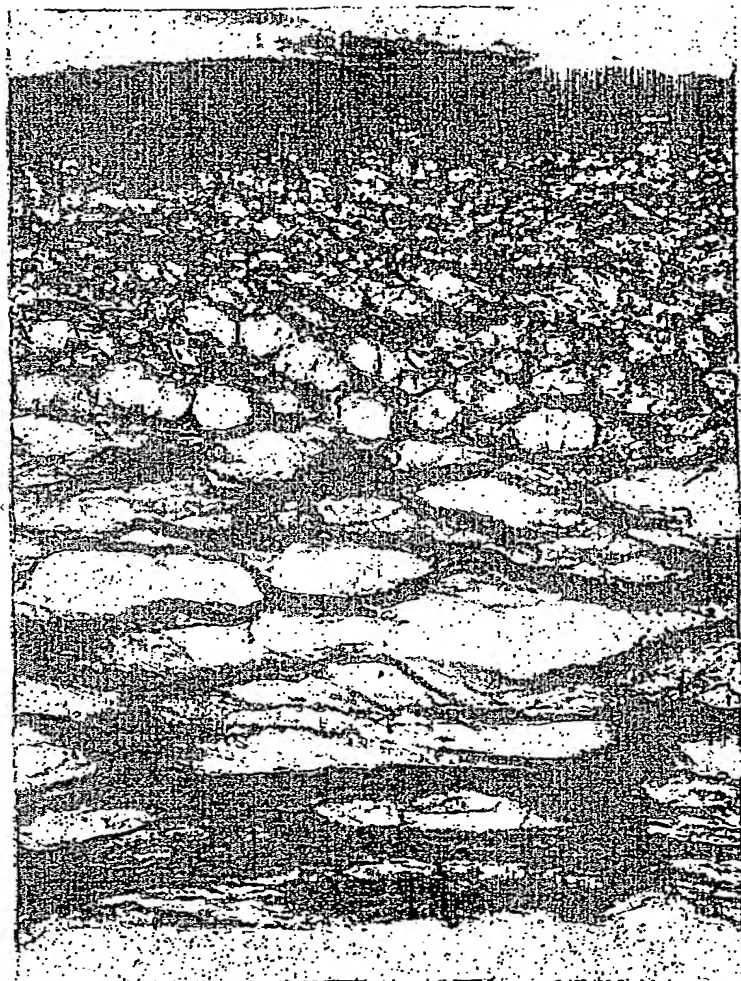


Fig. 1a

217

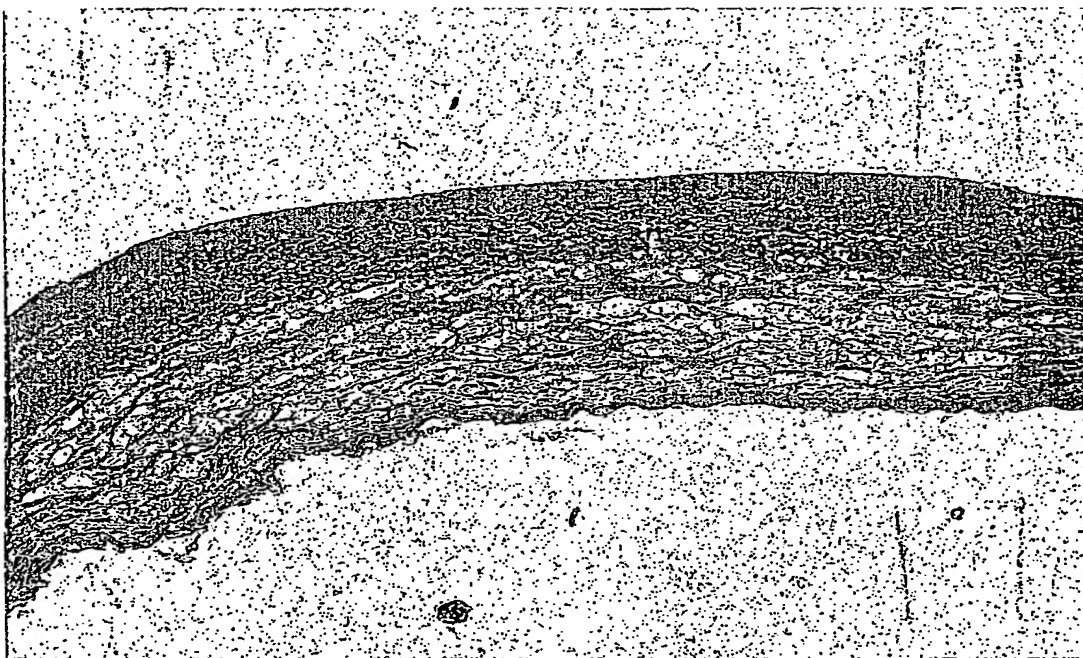


Fig. 1b

3/7



Fig. 2

4/7

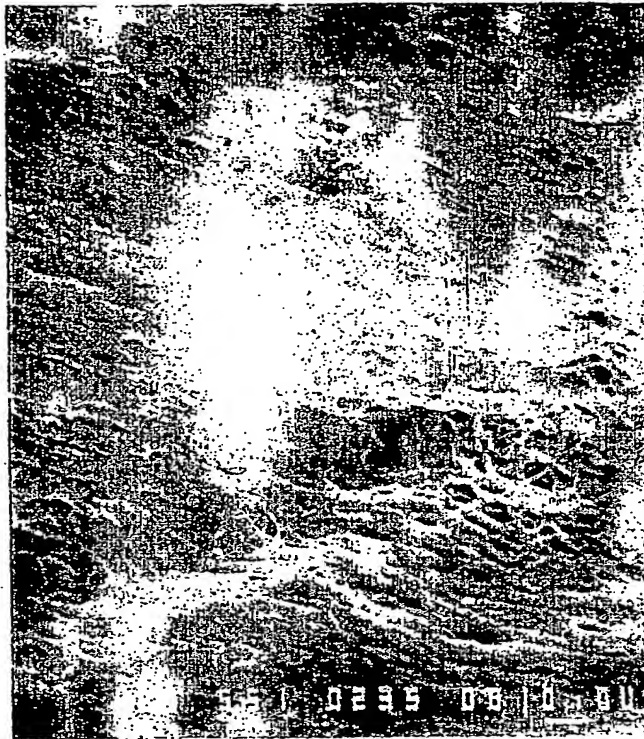


Fig. 3



517



Fig. 4a

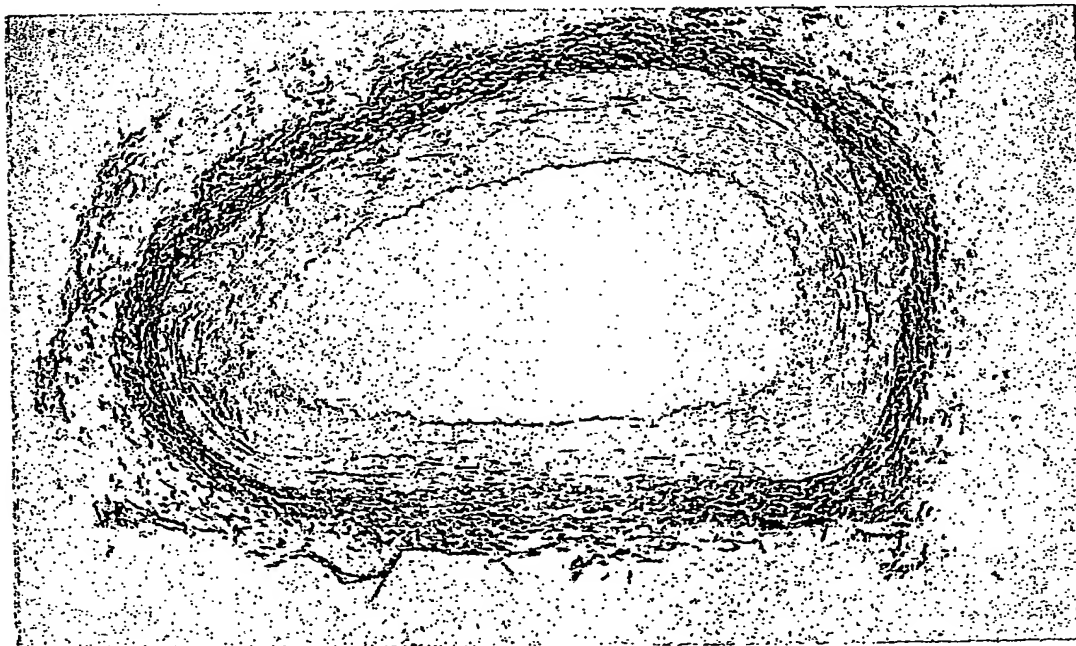


Fig. 4b

6/7

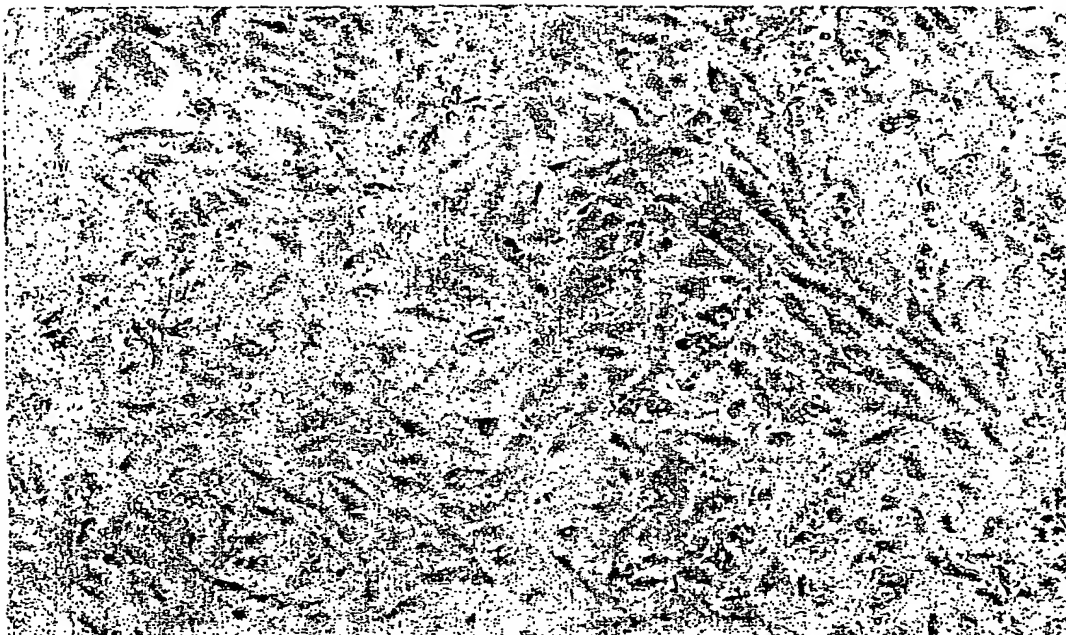


Fig. 5

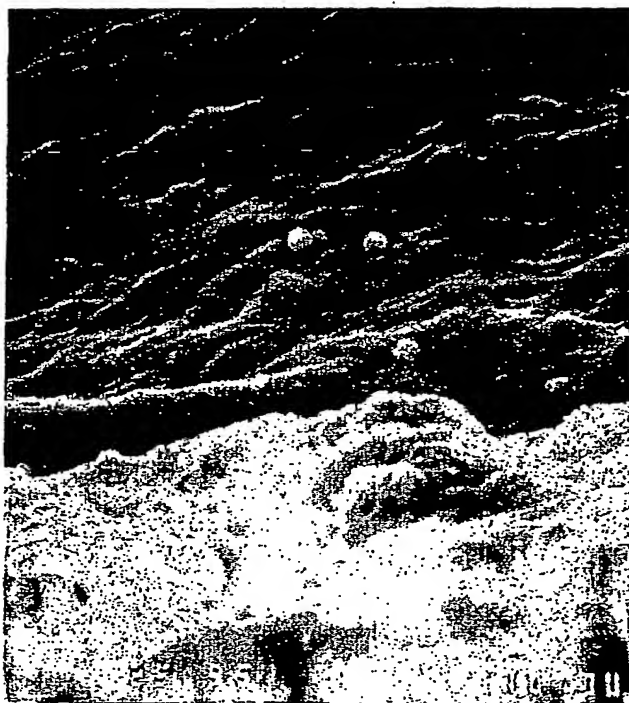


Fig. 6

717

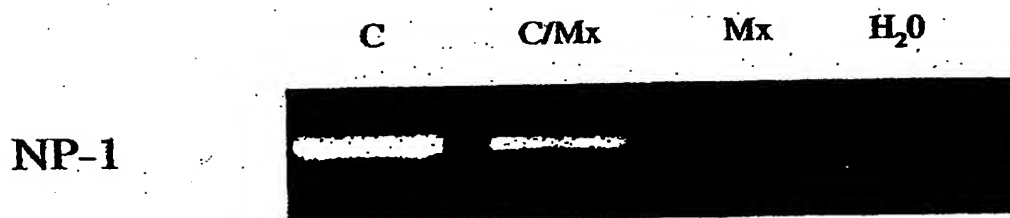


Fig. 7

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**